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# Assembly of the Kdp complex, the multi-subunit $K^+$-transport ATPase of *Escherichia coli*

Michael Gaßel [a], Annette Siebers [b], Wolfgang Epstein [c], Karlheinz Altendorf [a,*]

[a] *Universität Osnabrück, Fachbereich Biologie/Chemie, Abteilung Mikrobiologie, D-49069 Osnabrück, Germany*
[b] *MPI für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany*
[c] *Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637, USA*

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**Abstract**

Kdp, the high affinity ATP-driven $K^+$-transport system of *Escherichia coli*, is a complex of the membrane-bound subunits KdpA, KdpB, KdpC and the small peptide KdpF. The assembly of this complex was studied by the analysis of mutants that expressed two of the three large subunits and inserted them into the cytoplasmic membrane. In the strains that do not express KdpC or KdpA the other two subunits did not copurify on dye–ligand affinity columns after solubilization with non-ionic detergent. In the mutant lacking KdpB the other two subunits copurified under the same conditions. It is concluded that KdpC forms strong interactions with the KdpA subunit, serving to assemble and stabilise the Kdp complex. A structure in which KdpC could be one of the connecting links between the energy-delivering subunit KdpB and the $K^+$-transporting subunit KdpA is suggested by these data. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* Kdp-ATPase; Assembly; Subunit interaction; *Amber* mutation

## 1. Introduction

Kdp is an inducible high affinity potassium uptake system of *Escherichia coli* that belongs to the class of P-ATPases (for review see [1]). Kdp is a complex of the four inner membrane subunits KdpF, KdpA, KdpB, and KdpC that remains intact upon solubilization with non-ionic detergents and purification [2]. The genes coding for these four proteins are organized in the *kdpFABC* operon. Adjacent and overlapping with the *kdpC* gene is the *kdpDE* operon encoding the regulatory proteins: the KdpD sensor kinase and the KdpE response regulator [3–5]. It is believed that low turgor is the signal that activates KdpD to phosphorylate KdpE which stimulates transcription of the *kdpFABC* operon [6].

The Kdp complex appears to contain equimolar amounts of the three large subunits KdpA, KdpB, and KdpC [7,8] and seems to work as an oligomer [9]. Thus, the minimal stoichiometry is $A_2B_2C_2$ (Fig. 1), but little is known about the arrangement of the subunits in the KdpFABC complex. The largest 72 kDa KdpB subunit spans the membrane probably six times and forms a phosphointermediate during the catalytic cycle [10,11]. KdpB is homologous to the large subunit of other P-ATPases and shares the common key structures of this ATPase class [12,13], e.g. the ATP binding site and the highly con-

* Corresponding author. Fax: +49 (541) 969-2870; E-mail: Altendorf@biologie.uni-osnabrueck.de

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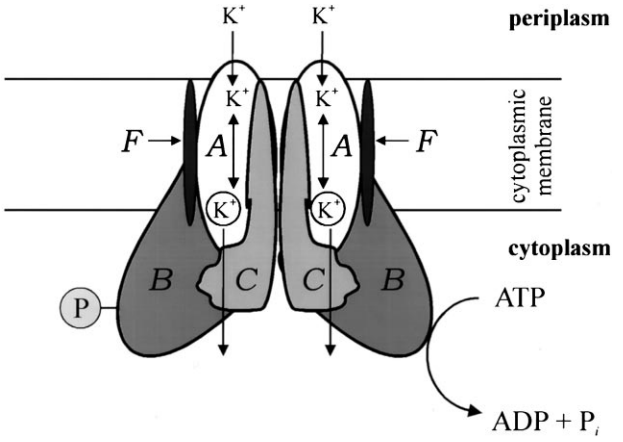



Fig. 1. Model of the Kdp-complex (modified according to [1,11]).

served DKTGT sequence with the putative phosphorylation site Asp307 [14]. The 59 kDa KdpA subunit is predicted to cross the membrane 10 times and is believed to be the subunit that binds and transports $K^+$ [15]. The structure reveals similarities to the S1-H5(P-loop)-S2 segment of eukaryotic and prokaryotic $K^+$-channels [16] from which it might be derived evolutionary [1]. The 20 kDa KdpC subunit probably crosses the membrane once close to the N-terminus with the C-terminal portion in the cytoplasm. Although KdpC is essential for the Kdp complex [7,17] its specific role is still unknown. The small 3 kDa peptide KdpF is not essential in vivo, since the growth behaviour of a deletion strain is comparable to that of the wild-type [18].

In this paper we have sought information on the assembly and structure of the Kdp complex by studying the effect of expressing only two of the three large subunits. The stability of the Kdp complex to non-ionic detergents indicates the existence of strong subunit interactions. The stability could arise from a few strong interactions, from a larger number of relatively weak interactions or from a combination of these possibilities. If the former is the case, then if any one of the subunits is not made the other two will remain associated. However, if only weak interactions are responsible for stability, loss of one subunit may result in dissociation of the other two. In this work we have used either chain-terminating mutations to eliminate expression of one essential Kdp subunit at time, or a plasmid carrying only the genes for two of the large subunits. The results show that when KdpC or KdpA is not made the other two subunits are no longer associated with each other, dissociating when fractionated on dye–ligand affinity columns. By contrast, loss of KdpB does not eliminate association of KdpA and KdpC during purification. Thus, both KdpA and KdpC are necessary to assemble a stable complex containing KdpB.

Table 1
*Escherichia coli* strains and plasmids

| Strain/plasmid | Relevant markers | Source/Ref. |
|---|---|---|
| TKA1000 | *kdp*$^+$ *Δatp706 nagA trk405 trkD1 thi rha lacZ* | [19] |
| TK2240 | *kdp*$^+$ *nagA trk405 trkD1 thi rha lacZ* | W. Epstein |
| TK2247 | *kdp*$^+$ *nagA nadA trkA405 trkD1 thi rha lacZ* | W. Epstein |
| TK2665 | *kdpB30 nagA trkD1 thi rha lacZ* | W. Epstein |
| TK2666 | *kdpC34 nagA trkD1 thi rha lacZ* | W. Epstein |
| TK2205 | *ΔkdpABC5 nagA trk405 trkD1 thi rha lacZ* | W. Epstein |
| F′100 | F′ *kdp*$^+$ | W. Epstein |
| pSR4 | *kdp*$^+$ *Ap*$^r$ | [14] |
| F′B30 | F′100 *kdpA*$^+$ *kdpB30 kdpC*$^+$ | W. Epstein |
| F′C34 | F′100 *kdpA*$^+$ *kdpB*$^+$ *kdpC34* | W. Epstein |
| pDD5-89 | *kdpA*$^+$ *Ap*$^r$ | W. Puppe |
| pMGkdpBC | *kdpB*$^+$ *kdpC*$^+$ in pJLA503-1 background | M. Gaßel |
| pJLA503 | $\lambda_{P_LP_R}$*−promoter cI*$^{857}$ *Ap*$^r$ | [25] |
| pJLA503-1 | $\lambda_{P_LP_R}$*−promoter cI*$^{857}$ *Ap*$^r$ | M. Gaßel |
| pUC19 | *Ap*$^r$ | |

## 2. Materials and methods

### 2.1. Materials

All chemicals used were of analytical grade. The triazine dye matrices Fractogel TSK AF-Red (dye: Procion Red HE-3B), Fractogel TSK AF-Blue (dye: Cibacron Blue F3-GA) and Fractogel TSK AF-Green (dye: Procion Green H4-G) were obtained from Merck (Darmstadt, Germany). DEAE-Sepharose CL-6B was purchased from Pharmacia (Freiburg, Germany).

### 2.2. Bacterial strains, plasmids, and growth conditions

*E. coli* strains and plasmids are listed in Table 1. All strains are derivatives of *E. coli* strain K12. The *kdp*$^+$ strain TKA1000 was grown in K0-media as previously described [19]. The *trkA*$^+$ strains TK2665/F′B30 and TK2666/F′C34 were grown in a medium containing 0.15 mM $K^+$. All cells were harvested at an optical density (610 nm) of about 1.0. TK2205/pMGkdpBC was grown in KML medium at 37°C to an optical density (610 nm) of about 0.8 and then shifted to a temperature of 40°C for 2 h.

### 2.3. Recombinant DNA techniques

The conditions for recombinant DNA manipulations and for transformation of *E. coli* cells were as described [20]. Treatment of DNA with restriction endonucleases, T4 DNA ligase, Klenow fragment, Tfl polymerase and calf intestinal phosphatase was performed following the protocols of the suppliers. DNA fragments were recovered from agarose gels using the Jetsorb kit from Genomed. Sequencing of DNA was performed by the dideoxynucleotide chain termination method [21] using the T7 sequencing system from Pharmacia. As a template, double stranded plasmid DNA denatured with NaOH was used as described in the protocol of the sequencing kit. For sequencing, the universal and reverse primers provided with the sequencing kit were used.

### 2.4. Construction of the strains

The amber-strains are derivatives of strain TK2247 [22] into which different *kdp* alleles were introduced by P1 cotransduction [23] with the *nadA* marker (15% cotransduction frequency). Strains that are homozygous diploid for the mutations in *kdpA*, *kdpB* and *kdpC* were created by introducing F′100 plasmids with the appropriate amber mutations. The *kdpB30* [24] is an amber mutation, while the *kdpC34* [7] mutation results in no detectable KdpC product, but is not suppressed by either *supE* or *supF* and is inferred to be either an *ochre*, *opal* or a frameshift mutation.

### 2.5. Construction of the plasmids

The plasmid pMGkdpBC was constructed using the PCR technique with pSR4 as template and with the following primers (introduced restriction sites are underlined and mentioned behind the primer, identical nucleotides are in italics, the position of the bp is set in square brackets):

B2: 5′ TC*ATGA*GTCGTAAACAACTGGCGGC 3′
(*Bsp*HI) [1697–1718]

B6: 5′ A*TCGAT*GTTGATCCCGCTC 3′
(*Cla*I) [2562–2545]

B7: 5′ *ATCGAT*AACCGCATGATCCGTAAAG 3′
(*Cla*I) [2858–2882]

B8: 5′ GGATCCACGGGTCATCAGCATC 3′
(*Bam*HI) [3417–3397]

B9: 5′ *GGATCC*CTGACCACCTTCAGCAT 3′
(*Bam*HI) [3413–3435]

C18: 5′ *GTCGAC*AATGCCGGACGTAATCCACT 3′
(*Sal*I) [3782–3758]

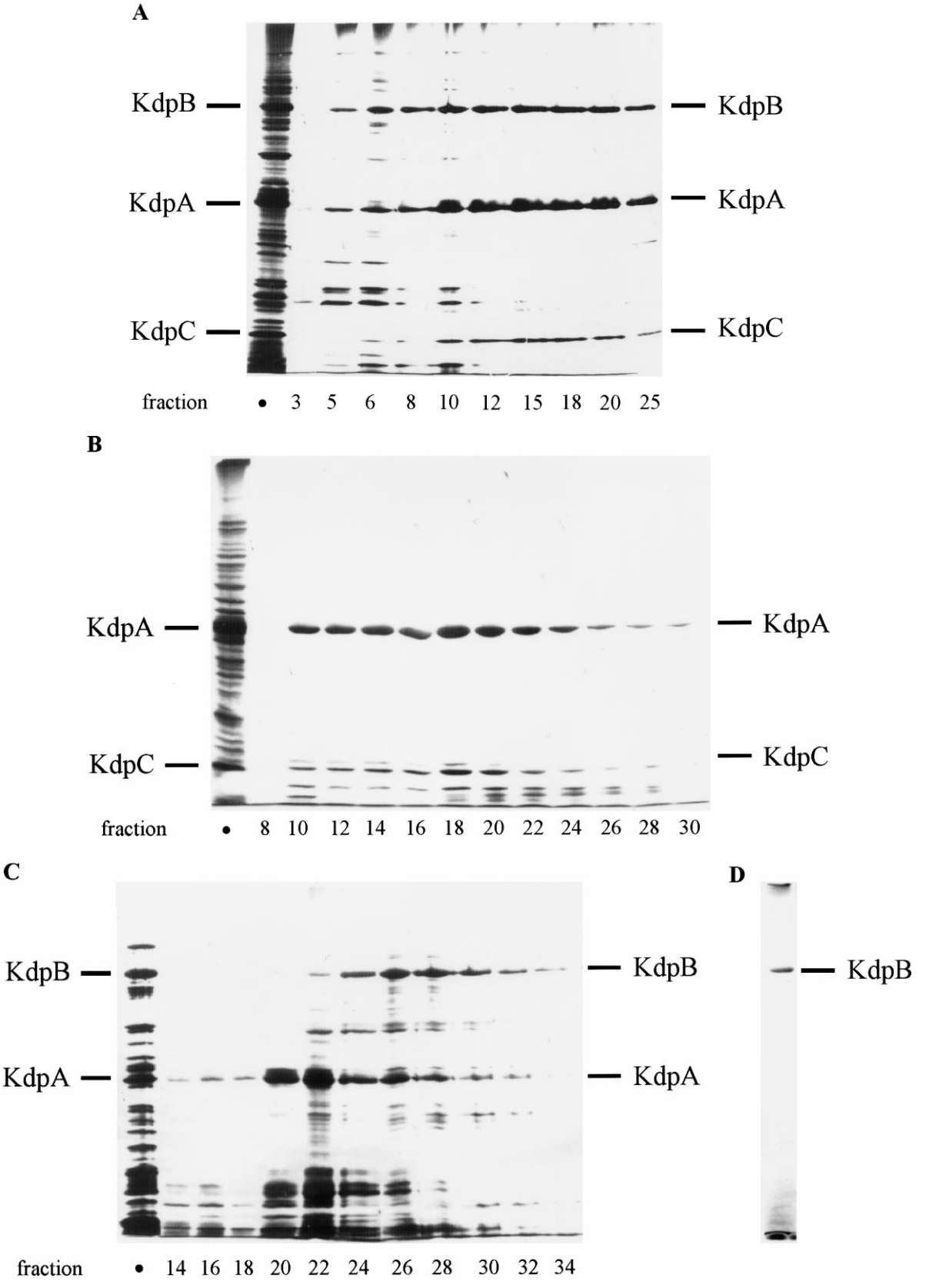
C17′: 5′ GTCG*AC*ATTTATCTTTCTGTTATTG 3′
(*Sal*I) [3778–3801]

C6: 5′ GAA*TTC*GTTATTCATCAA 3′
(*Eco*RI) [4330–4116]

The PCR products generated with primer-pairs B2/B6, B7/B8, B9/C18 and C17′/C6 were cloned blunt end in pUC19 and sequenced. Using the silent restriction sites, which were introduced by the PCR primers, the four parts were joined and the whole construct was cloned in pJLA503-1, a modified pJLA503 vector [25], in which the *Sal*I restriction site of the multiple cloning site was filled in with Klenow.

### 2.6. Protein purification and ATPase activity of the Kdp complexes

Membranes were prepared by passage of cells through a Ribi press, washed twice in EDTA con-


A
KdpB
KdpA
KdpC
KdpB
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fraction • 3 5 6 8 10 12 15 18 20 25
B
KdpA
KdpC
KdpA
KdpC
fraction • 8 10 12 14 16 18 20 22 24 26 28 30
C
KdpB
KdpA
KdpB
KdpA
fraction • 14 16 18 20 22 24 26 28 30 32 34
D
KdpB

Fig. 2. Purification of Kdp subunits from *kdp* wild-type strain and *kdp* chain-terminating mutants. Membranes of TKA1000 *kdp*$^+$ (panel A), TK2665/F′B30 (panel B) and TK2666/F′C34 (panels C and D) were solubilized with Aminoxid WS 35. The membrane extract was passed through a DEAE-Sepharose CL-6B column and the non-adsorbed proteins were loaded on a Fractogel TSK AF-Red column and eluted with a 0–1.5 M KCl gradient. The salt gradient was modified for TK2666/F′C34 (panel C) in order to optimize the separation between KdpA and KdpB. A prewashing step with 0.35 M KCl (fractions 1–20) gave better results although smearing of KdpA during this pretreatment and during the continuation of the gradient up to 1.5 M KCl could not be completely prevented. When the membrane extract of TK2666/F′C34 was applied directly on a Fractogel TSK AF-Blue column (panel D), pure KdpB protein could be eluted with a 0–1.5 M KCl gradient. The silver stained SDS polyacrylamide gels depict the protein composition of the membrane extracts (labelled with a closed circle in panels A, B, C) and of the column fractions of the corresponding Fractogel TSK AF-Red column (panels A, B, C), identified by number. In panel D the Fractogel TSK AF-Blue fraction pool for KdpB is shown. The positions of the KdpB, KdpA and KdpC subunits are indicated.
←

taining buffer of low ionic strength and solubilized with the non-ionic detergent Aminoxid WS 35 [19]. Binding studies of mutant Kdp complexes to different triazine dye matrices were performed on an analytical scale (see data in Table 2). 100–150 mg of membrane protein were solubilized [2] and approximately 20 mg of the diluted extract were directly applied to a red, blue or green Fractogel TSK AF column (1×2 cm; volume 1.6 ml). Unbound material was washed off and elution of the columns was performed with a linear gradient of 0–1.5 M KCl in basal buffer. On the preparative scale (see data in Fig. 2) the two column procedure with DEAE-Sepharose CL-6B and dye–ligand affinity chromatography on Fractogel TSK AF-Red [2] was applied unless otherwise stated. The ATPase activity of the Kdp complexes were measured as described in Siebers et al. [19].

### 2.7. Analytical procedures

Determination of protein concentrations was carried out as described [26]. Proteins were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Lugtenberg et al. [27]. Gels contained 12% (w/v) acrylamide and 0.32% (w/v) bisacrylamide. Subsequently, the gels were stained with silver [28]. The electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose membranes (0.45 μm) and immunodetection of Kdp with anti Kdp antiserum was carried out as described in Siebers et al. [19].

## 3. Results

For this study of the association/dissociation behaviour of Kdp partial complexes we screened different mutants with chain terminating mutations in one of the three essential *kdpABC* genes. Immunoblotting analysis of whole cell lysates and cytoplasmic membranes was done to confirm the absence of an intact product of the defective gene and to verify the effective expression of wild-type genes and the membrane insertion of the wild-type gene products. By these criteria, the *kdp* mutant alleles *B30* and *C34* were

Table 2
Association character of Kdp subunits after solubilization and different dye–ligand chromatographic separations[a]

| *E. coli* strain | *kdp* genotype | Fractogel TSK Affigel | | |
|---|---|---|---|---|
| | | red | blue | green |
| TKA1000 | *kdpABC*$^+$ | ABC | – | (ABC) |
| TK2665/F′kdpB30 | *kdpAC*$^+$ *kdpB*$^-$ | AC | (AC) | (AC) |
| TK2666/F′kdpC34 | *kdpAB*$^+$ *kdpC*$^-$ | (A+B) | B [A] | (A+B) |
| TK2205/pMGkdpBC | *kdpBC*$^+$ *kdpA*$^-$ | B+C | n.d. | n.d. |

[a]Detergent solubilized membrane proteins of the strains were loaded on columns at low NaCl concentration, and eluted with a linear salt gradient. The association state is indicated as follows: ABC, the entire Kdp complex; AC, a complex of KdpA and KdpC; A+B and B+C, two unassociated subunits; [A], subunit does not bind to the column; –, no binding; symbols in parentheses, weak binding; n.d., not determined.

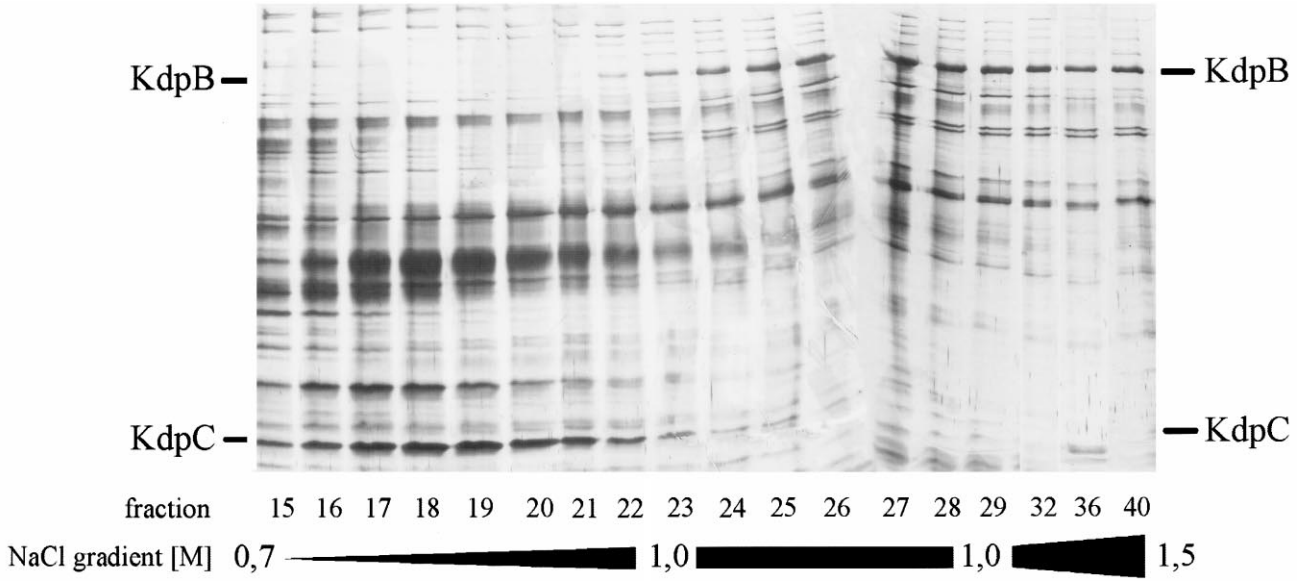


Fig. 3. Purification of Kdp subunits from the strain TK2205/pMGkdpBC. Membranes of TK2205/pMGkdpBC were solubilized with Aminoxid WS 35. The membrane extract was loaded on a Fractogel TSK AF-Red column and eluted with a 0–1.5 M KCl gradient. The silver stained SDS polyacrylamide gel depicts the protein composition of the Fractogel TSK AF-Red column fractions identified by number. The positions of the KdpB and KdpC subunits are indicated.

chosen for further studies. None of everted vesicles derived from these strains had measurable $K^+$-stimulated ATPase activity supporting the view that all of the Kdp components are indispensable for a functional ATPase complex.

Membranes of the selected strains were solubilized and subjected to dye ligand affinity chromatographic separations [2] to test subunit association behaviour (Table 2). This method proved to be useful since the affinity matrices had different binding characteristics towards the intact Kdp complex, the KdpAC partial complex and individual Kdp subunits (Table 2).

Subsequent studies were performed on a preparative scale by combining ion exchange and dye–ligand affinity chromatography. Analysis of proteins in extracts of the *kdpB30* and *kdpC34* mutant was easily performed since the intact subunits were expressed at a high rate and were reasonably stable during purification. Fig. 2 shows the Fractogel TSK AF-Red column fractionation of a $Kdp^+$ membrane extract (panel A) along with similar samples derived from the two mutant strains (panels B an C). The elution profile of KdpA and KdpC, which were derived from the *kdpB30* mutant strain (Fig. 2B), is similar to the parallel elution of all three proteins of the wild-type strain (Fig. 2A). By contrast, in the absence of KdpC, KdpA elutes before KdpB (Fig. 2C). Unfortunately, a complete separation of KdpA from KdpB could not be achieved on the AF-Red matrix due to a characteristic smearing of the KdpA protein on columns. The failure of KdpA to give distinct elution peaks is probably based on the formation of heterogeneous micellar structures caused by the extreme hydrophobicity of this protein. Indeed, the KdpA derived from the multicopy plasmid pDD5-89 (*kdpA*$^+$ *ΔkdpBC*, constructed by W. Puppe) showed the same characteristic smearing in the absence of the other Kdp subunits. A different dye column, Fractogel AF-Blue, separates them completely because the KdpA protein flowed through during the loading step so that pure KdpB was obtained upon salt elution (Fig. 2D; Table 2).

Attempts to use a *kdpA amber* mutation in the same way failed, because all six such mutations tested expressed the other subunits at a only low level and there was considerable proteolysis of the KdpB subunit. Therefore, we constructed the plasmid pMGkdpBC carrying the wild-type genes of *kdpB* and *kdpC* behind the temperature inducible $\lambda_{P_L P_R}$-promoter. Strain TK2205 (*ΔkdpFABC*) transformed with the plasmid pMGkdpBC (*kdpBC*$^+$) was able to express KdpB and KdpC at a high

rate. Again, share of KdpB was subject to proteolytic degradation. Membrane extract of this strain was applied on TSK AF-Red matrix and protein were eluted with a KCl gradient (Fig. 3). As can be seen, KdpC eluted before KdpB, although there are three fractions of coelution (fractions 23–25) albeit none of these contain large amounts of either subunits.

## 4. Discussion

The assembly of the three essential subunits KdpA, KdpB and KdpC of the Kdp ATPase of *E. coli* is the subject of this investigation. We have used chain-terminating mutations in the *kdpABC* genes to examine the fate of the expressed subunits when one of the large proteins is not present. These mutants are expected to express shorter fragments, but no truncated subunit could be visualised either by staining or with specific polyclonal antibodies. The results indicate that when KdpC is present, it associates with KdpA to form a partial complex stable to fractionation on ion exchange and affinity columns. However, when KdpC is not present the other two subunits fall apart, showing a different pattern of elution from the Fractogel AF-Red column (Fig. 2C) and complete separation on the Fractogel AF-Blue column (Fig. 2D). When KdpA is not made, the other two subunits bind to the Fractogel TSK AF-Red column, but upon elution of the column, they appear in different fractions (Table 2, Fig. 3), indicating that these two subunits do not form a partial complex either.

These results indicate that in the Kdp complex only the KdpA and KdpC subunits interact strongly. Whatever interactions KdpB has with KdpA and KdpC, they are not strong enough to withstand the relatively mild conditions of solubilization with non-ionic detergents and fractionation on ion exchange and affinity columns. We infer from the functions of KdpB and KdpA [11] that they interact during transport by Kdp, since the former is the site of energy coupling to ATP while the latter appears to be the site of $K^+$-binding and transport [15]. Thus, at least parts of the internal region of KdpB ought to interact with parts of KdpA or KdpC, but the interactions seemed to be energetically weak. This model fits well with the suggestion that KdpB and KdpA had separate evolutionary origins, and subsequently associated to form a single complex [29,11]. That KdpC might have been associated with the complex together with KdpA is suggested by the formation of the KdpAC partial complex. Close physical contact between subunits is also the prerequisite for functional communication in terms of transmission of conformational changes from the energy-providing subunit KdpB to the energy-consuming transport subunit KdpA. Consequently, the role of KdpC might be that of a structural and functional mediator in the Kdp complex although it is more strongly associated with the KdpA subunit. The structural role of KdpC is supported by the fact that it is essential [7,17] while a functional role is implied by the labelling of both KdpB and KdpC with 2-azido-ATP [30].

The chain-terminating mutants are useful as well in preparing Kdp subunits in non-denatured and purer form. Previously, single Kdp proteins were separated after dissociation of the complex by sodium dodecyl sulfate [19]. Non-denaturing affinity-purified KdpB protein or KdpAC partial complex will be useful for functional reconstitution studies investigating the roles of the different Kdp components in the complex or for crystallization attempts.

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